Chinese Science Bulletin 2006 Vol. 51 Supp. I 117—123

DOI: 10.1007/s11434-006-8215-y

Systematic position of *Myrtama* Ovcz. & Kinz. based on morphological and nrDNA ITS sequence evidence

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Received July 20, 2005; accepted January 16, 2006

Abstract *Myrtama* is a genus named from *Myricaria elegans* Royle in the 1970's in terms of its morphological peculiarities. The establishment of this genus and its systematic position have been disputed since its inception. ITS sequences from 10 species of Tamaricaceae are reported, and analyzed by PAUP 4.0b8 and Bayesian Inference to reconstruct the phylogenies. A single ITS tree is generated from maximum parsimony and MrBayes analyses, respectively. The molecular data set shows strong support for *Tamarix* and *Myricaria* as monophyletic genera, and *Myrtama* as a sister group to the genus *Myricaria*. Based on morphological differences, a single morphological tree is also generated, in which two major lineages existed but *Myrtama* is a sister group to *Tamarix*, rather than *Myricaria*. The evidence from DNA sequences and morphological characters supports that *Myicaria elegans* should be put into neither *Myricaria* nor *Tamarix*, but kept in its own monotypic genus.

Keywords: ITS sequence, *Myrtama* Ovcz. & Kinz, *Myricaria elegans* Royle, Tamaricaceae.

Tamaricaceae are a relatively small family of 3—5 genera and about 100 species distributed in temperate and subtropical Africa and Eurasia on salty or dry areas of deserts, steppes, sandy shores, and along rivers[1,2]. There are two tribes, Reaumurieae (*Hololachna*, *Reaumuria*) and Tamariceae (*Myricaria*, *Myrtama*, *Tamarix*), in which *Tamarix* is the largest genus in the family containing approximately 54 species[3], *Myricaria* contains about 10 species of shrubs (some prostrate) ranging from Europe (one species) to central Asia in mountainous areas up to 6500 m[4], and *Reaumuria* is a genus of about 12 small shrub species ranging from Europe to central Asia in arid or semi-arid areas. Among all the genera of Tamaricaceae, *Myrtama* and *Hololachna* are two small taxonomically problematic genera[5].

Myrtama is a relatively new genus named from *Myricaria elegans* Royle which was originally described from the Kunawar region of the western Himalayas[6] and grows in riversides and lakefronts at an altitude of 3000—4300 m in Xinjiang, Xizang of China and Kashmire areas. The species has ten stamens, flat leaves, and sessile stigmas, so placement in *Myricaria* was reasonable, but the stamens are distinct, which is why Baum transferred *Myricaria elegans* to *Tamarix*, giving it the name *T. ladachensis* because of the preoccupation of the epithet *elegans* under *Tamarix*, and noted that 'this is the only species of *Tamarix* with flat leaves and beaked seeds'[3]. In 1977, Ovczhinnikov and Kinzikaeva considered the species intermediate to *Myricaria* and *Tamarix*, and placed it in its own genus, *Myrtama*[7]. Only one year later, Qaiser and Ali[8] also named *Myricaria elegans* as a novel genus, *Tamaricaria* (synonymous with *Myrtama*). Zhang *et al*.[9] investigated pollen morphology and also supported to put it in its own genus, *Myrtama*. Zhang and Zhang[4], Tang[10] and Xi[11] also discussed the taxonomic status of *Myrtama elegans* with different morphological evidence and agreed with its original placement in *Myricaria*, although they noted the existence of unique characteristics. Wu *et al*.[12] considered that *Myrtama* (= ×*Tamaricaria*) was the hybrid genus originated from *Tamarix* × *Myricaria*.

Till now, the taxonomy and systematics position of *Myricaria elegans* is still in dispute. In order to take light on it, Gaskin *et al.* [5] have inducted molecular analysis by sequencing 18S nuclear rDNA and plastid *rbc*L sequence, and got the result that supports the establishment of the genus *Myrtama*. Here we just used another fragment, internal transcribed spacer sequence (ITS) of nuclear ribosomal DNA, in combination with morphological evidence to clarify the establishment and placement of the genus *Myrtama*.

1 Materials and methods

1.1 Taxon sampling

Ten species representing four genera of the Tamari-

caceae were collected from natural populations or cultivated plantings. Vouchers are deposited in the herbarium of the Turpan Eremophytes Botanical Garden, Xinjiang Institute of Ecology and Geography, the Chinese Academy of Sciences, except *Myrtama elegans* which is deposited at Missouri Botanical Garden (MO). The sampled taxa and their GenBank sequence accession numbers are listed in Table 1.

1.2 DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from silica-gel dried or fresh leaves with a CTAB method[13]. Universal primers "ITS3" and "ITS4" of White[14], P1 and P2 were used for amplification and sequencing. P1 and P2 were designed according to rRNA gene sequence of *Oryza sativa*[15]. P1 is located 48—23 bp upstream from the 3′ end of the 18S rRNA gene (5′ AGA AGT CGT AAC AAG GTT TCC GTA GG 3′), and P2 is 15—39 bp downstream from the 5′ end of 5.8S gene (5′ GAT GCG AGA GCC GAG ATA TCC GTT G 3′)[16]. Double-stranded DNA products including complete sequences of ITS-1, 5.8S rDNA and ITS-2 were generated by primer pair P1 and "ITS4". The PCR products were purified by Wizard PCR preps DNA purification system (Promega), and then sequenced directly on an ABI 377 DNA Sequencer.

1.3 Alignment and analysis of the sequence data

Reaumuria songarica was designed as the outgroup in this study. All ITS sequences were aligned using the software Clustal X[17]. The basic statistics, including nucleotide frequencies, pairwise distances and variable sites were completed by Mega 2.0[18] and PAUP 4.0b8[19].

The aligned sequences were analyzed through the program PAUP 4.0b8 and MrBayes version 2.01[20]. Phylogenetic analyses were performed using maximum parsimony method, with branch-and-bound search and furthest addition sequence options. Gaps were treated as missing data. Support for topology was estimated with the bootstrapping analysis in PAUP 4.0b8 using 1000 replicates. By using a hierarchical likelihood ratio test approach implemented in the program MODELTEST 3.0[21], A GTR+G+I model of DNA substitution that best fits the data for bayesian analysis was selected. In the Bayesian analysis, MrBayes settings were: run for 130000 generations (ngen = 130000); save the current tree every 100 generations (sample freq =100), run four simultaneous MCMC chains, ignore the first 300 trees (burnin = 300), and generate posterior probabilities of the trees.

1.4 Morphological and combined data analysis

Based on extensive specimen studies and the literature[5,7—9,22], we selected 16 stable morphological characters for analysis (Table 2). *R. songarica* was again designated as outgroup. In the morphological character matrix (Table 3), 7 characters were treated as binary and 9 were coded as multistate. Parsimony analysis was conducted using the heuristic and branch-and-bound algorithm of PAUP 4.0b8 with multistate characters treated as unordered. The distribution of nonhomoplasious and homoplasious characters was shown on the most parsimonious tree.

The data from ITS sequences and morphology were combined without weighting. Data were analyzed with PAUP 4.0b8 using the branch-and-bound and heuristic search options with gaps treated as missing data. Bootstrap values were used to assess the robustness of the estimated phylogenetic tree. Each of these analyses was based on 1000 replications.

Table 1 The origin of materials and GenBank accession number of ITS sequences

Genus	Species	Locality	Voucher	GenBank accession No.
Tamarix	*T. ramosissima*	Turpan Eremophytes Botanic Garden	ZDY-0019013	AY 207481
	T. leptostachys		ZDY-0019017	AY 207489
	T. elongata		ZDY-981041	AY 207483
	T. hispida		ZDY-981045	AY 207482
	T. chinensis		ZDY-981046	AY 207484
Myrtama	*M. elegans*	Xizang, China	Al Shehbaz 8433(MO)	AY 207488
Myricaria	*M. laxiflora*	Beijing Botanic Garden	ZDY-981101	AY 207486
	M. bracteata	Da He Yan, Xinjiang	ZDY-980701	AY 297487
	M.alopecuroides	Kazakstan	Wang Jian Feng 10 (USDA-GSWRL-BW)	AF 484746
Reaumuria	*R. songarica*	Turpan Eremophytes Botanic Garden	ZDY-0019019	AY 207485

Table 2 Morphological characters used in the cladistic analysis

1	Leaf form: columned (0), subulate or scalelike (1), flat (2)
2	Flowers: solitary (0), aggregated in inflorescences (1)
3	Each petal exhibiting a pair of scalelike appendages inside at the base (0), absent (1)
4	Inflorescences: in racemes (0), in panicles (1), in racemes of vernal inflorescence and panicles of aestival inflorescence (2)
5	Hypogynous disk: absent (0), present (1).
6	Androecium: 6－8(0), 4－5 (1), 10 (2)
7	Stamen: free (0), coherent at their bases (1), or in bundles (2)
8	Gynoecium: stylate(0), non-stylate (the stigmas sessile) (1)
9	Anther: extrorse (0), or introrse (1)
10	The mesh size of pollen grains: mesh absent (0), coarse mesh (1), slender mesh (2)
11	The sculpturing pattern of pollen grains: poriferous (0), reticulate (1), areolate and not perforated (2)
12	The sculpturing pattern of pollen grains: regular (0), irregular (1)
13	Seeds morphology: pappus cover all the seeds (0), pappus cover half or apex of the seeds (1), pappus only cover coma (2)
14	The coma: absent (0), almost absent (only 0.4 mm long) (1), present (2)
15	Pappus covers all the coma, nearly sessile pappus (0), pappus covers from bottom of the coma, nearly almost sessile pappus (1), pappus covers the upper part of the coma, nearly stipitate pappus (2)
16	Endosperm: present (0), absent (1)

Table 3 Data matrix for morphological characters used in this analysis (including the outgroup *Reaumuria songarica*)

Myricarialaxiflora	1111022100202221
M.bracteata	1111022100202221
M. alopecuroides	1111022100202221
Myrtama elegans	2111021111112111
Tamarix hispida	1110110012101001
T. ramosissima	1112110011101001
T. chinensis	1112110012101001
T. leptostachys	1112110011101001
T. elongata	1111110011101001
Reaumuria songarica	000?000000000000

2 Results

2.1 ITS data

After alignment, there were 732 bp in the matrix; 241 are variable and 151 are parsimoniously informative. The percentage of variable sites was 32.9%, and the percentage of phylogenetically informative sites was 20.6%. The size of the ITS segment was 578－586 bp in *Tamarix*, 605 bp in *Myricaira* and 593 bp in *Myrtama*. The average base frequency within Tamaricaceae was as follows: T: 20.9; C: 28.7; A: 20.9; G: 29.5. The average G+C content was 58.2%. The G+C content of the *Tamarix* specimens varied in the range of 54%－63.5% *vs*. 50.4%－52.8% *Myricaria*. In *Myrtama elegans*, the G+C content was 51.7% (Table 4).

Uncorrected sequence divergence ranged from 1.5% to 21.6% between ingroups of Tamariceae. The sequence divergence between *Myrtama elegans* and species in *Myricaria* ranged from 5.1% to 6.1% and from 20.4% to 21.6% between *M. elegans* and the *Tamarix* species (Table 5).

2.2 Sequence analysis through the maximum parsimony (MP) method

A single most parsimonious tree of 325 steps was obtained (Fig. 1, CI = 0.912, RI = 0.895). The tree shows two major subclades with almost equal bootstrap support, one comprising five species of *Tamarix*, the other including *Myrtama* and three species of *Myricaria.* The *M. laxiflora-M. apoluroides-M. bracteata* clade is well supported by a bootstrap value of 99%, with *Myrtama elegans* as sister group (100% bootstrap value).

2.3 Sequence analysis through Bayesian Inference (BI) method

Bayesian Inference generated a tree with identical topology of that by MP analysis (Fig. 1, Ln likelihood = –2411.720290). There was also strong support for the

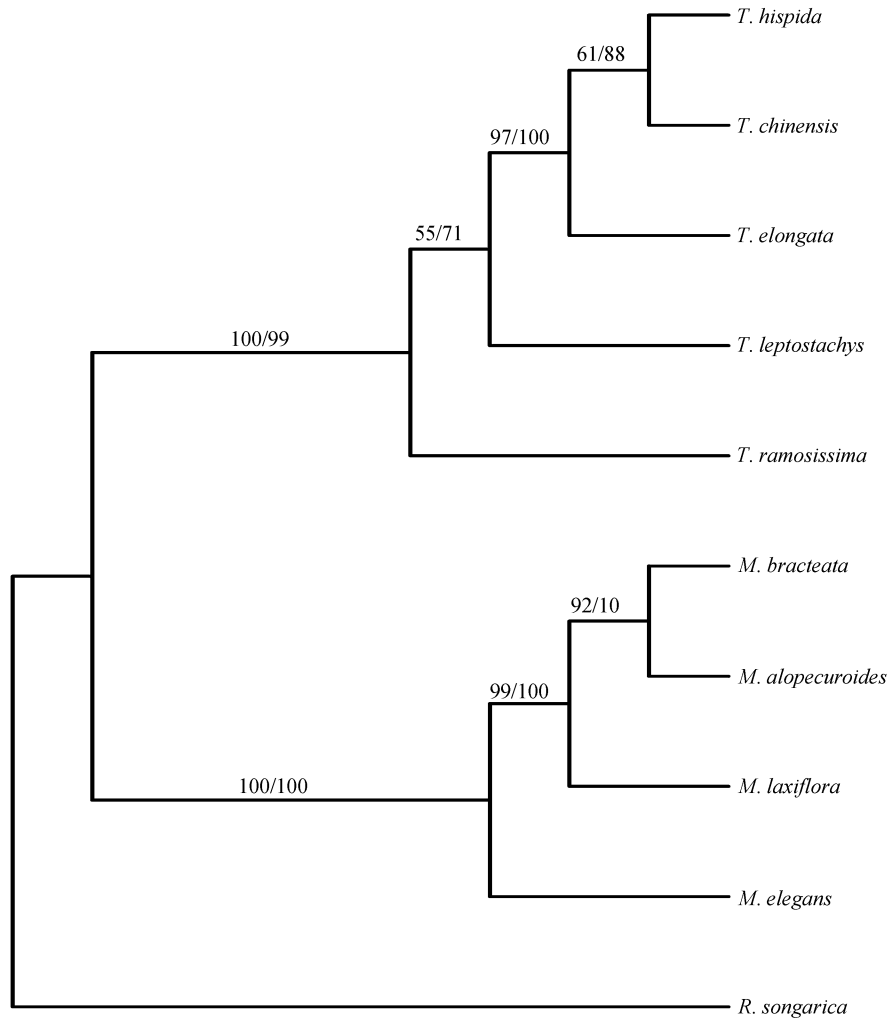

Fig. 1. The phylogenetic tree of Tamaricaceae based on ITS sequences. The numbers above branches indicate bootstrap values and posterior probabilities using PAUP analysis and Bayesian Inference method, respectively.

Table 4 Size, nucleotide frequencies and G+C content of ITS of 10 species from Tamaricaceae

	Size (bp)	G+C (%)	N (T)	N (C)	N (A)	N (G)
T. leptostachys	586	63.5	18.4	31.6	18.1	31.9
T. ramosissima	586	54.0	19.3	30.7	18.3	31.7
T. hispida	585	61.7	19.5	30.4	18.8	31.3
T. elongata	586	62.3	18.8	30.9	18.9	31.4
T. chinensis	578	62.2	19.2	30.4	18.5	31.8
M. elegans	593	51.7	25.6	25.1	22.6	26.6
M. laxiflora	605	52.8	23.0	26.8	24.3	26.0
M. bracteata	605	50.4	24.5	25.3	25.1	25.1
M. alopecuroides	605	50.9	24.7	23.5	24.5	27.4
R. songarica	562	63.2	16.4	31.7	20.5	31.5

monophyly of *Tamarix* (99%, posterior probabilities value, same thereafter), *Myrtama-Myricaria* (100%) and *Myricaria* (100%).

2.4 Morphological data

A single most parsimonious tree of 26 steps with a consistency index of 0.962 was yielded. The tree shows two major lineages, one comprising three species of *Myricaria*, the other including five species of *Tamarix* and *Myrtama elegans*, in disagreement with ITS trees (Fig. 2). Within the *Myricaria* clade, *M. laxiflora* + *M. alopecuroides* + *M. bracteata* are a monophyletic group

Table 5 Pairwise distances between taxa of Tamaricaceae and outgroup[a)]

	1	2	3	4	5	6	7	8	9	10
T. leptostachys	–	0.025	0.028	0.018	0.025	0.193	0.198	0.206	0.211	0.211
T. ramosissima	10	–	0.028	0.028	0.015	0.196	0.191	0.198	0.204	0.204
T. hispida	11	11	–	0.020	0.018	0.204	0.201	0.209	0.214	0.214
T. elongata	7	11	8	–	0.023	0.196	0.201	0.209	0.214	0.214
T. chinensis	10	6	7	9	–	0.206	0.204	0.211	0.216	0.216
R. songarica	76	77	80	77	81	–	0.242	0.254	0.262	0.249
M. laxiflora	78	75	79	79	80	95	–	0.015	0.025	0.051
M. bracteata	81	78	82	82	83	100	6	–	0.015	0.061
M. alopecuroides	83	80	84	84	85	103	10	6	–	0.056
M. elegans	83	80	84	84	85	98	20	24	22	–

a) Total character differences are indicated below diagonal, and mean character ones above.

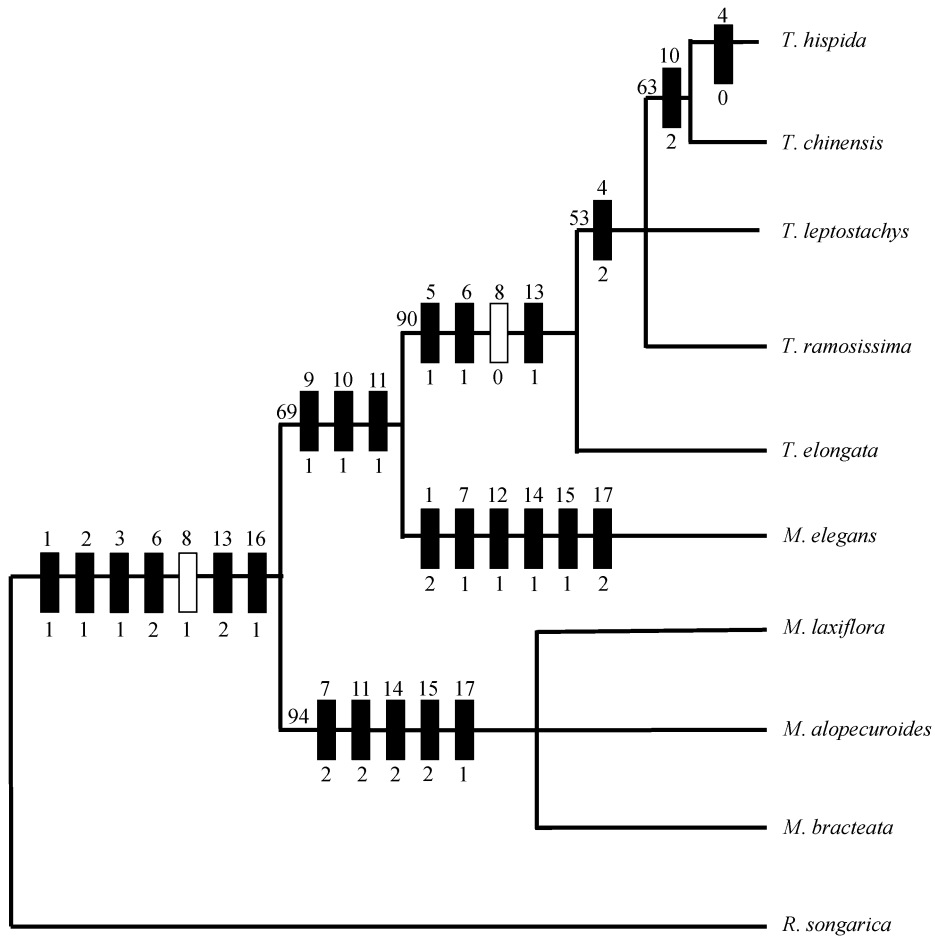

Fig. 2. Single most parsimonious tree for the Tamaricaceae based on morphological characters indicated in Table 3 with *Reaumuria soongarica* as the designated outgroup. Multistate characters treated as unordered. Length is 26 steps, with CI = 0.962, RI = 0.969. Bootstrap values are indicated at base of each clade. The distribution of morphological characters is shown with boxes. Character numbers are indicated above boxes and characters states indicated below boxes.

supported by 4 characters and a high bootstrap value (94%). The latter clade is well supported by three characters, in which five species of *Tamarix* formed a well supported clade (90%), and *Myrtama elegans* is the sister group of these *Tamarix*, and as a clade they are supported by three characters and a bootstrap value of 67%. The bootstrap support for the *Tamarix* and *Myricaria* clades and each clade within the two genera re-

mained the same as in the heuristic analysis.

2.5 Combined data

An analysis based on the combined data set from ITS and morphology with branch-and-bound search option, generated a single most parsimonious tree of 361 steps (CI = 0.900). The topology of the tree is all the same as that in the ITS trees (Fig. 1) in having two major lineages. The *Tamarix* clade, and the sister-group relationship between *Myricaria-Myrtama*, are reinforced with a bootstrap value of 100%. When using heuristic searches, the analysis yielded a single most parsimonious tree with bootstrap support values similar to that in the above tree.

3 Discussion

The taxonomic position of *Myrtama* within the Tamaricaceae has been debated for decades. Some have supported the idea of placing *Myrtama elegans* into *Myricaria* with the name *Myricaria elegans*[4,6,10,11,22], some considered it more reasonable to put it into *Tamarix* with the name *T. ladachensis*[3], others agreed to the establishment of a new genus, *Myrtama,* with the epithet *Myrtama elegans*[5,7−9,22], and others also agreed to the establishement of the genus *Myrtama*, but considered it a hybrid genus[12]. In the present ITS sequence analyses, we found that the fragment length, the G+C contents and the number of variable sites of *Myrtama elegans* were different from those of *Tamarix* and *Myricaria*, and just in the middle range of the variation. The relative distance between *M. elegans* and *Myricaria* species ranged from 5.1% to 6.1%, much higher than that within the genus *Myricaria* (1.5%−2.5%), and ranged from 20.4% to 21.6% between *M. elegans* and *Tamarix* species, also much higher than that within the genus *Tamarix* (1.5%−2.8%) (Table 5). In addition, the separate analyses of ITS and combined data all describe two major lineages, one being the *Tamarix* clade, the other being the *Myrtama-Myricaria* clade, with bootstrap values of 100%; while the morphological tree generated different topology: *Myricaria,* and *Myrtama-Tamarix* clades. We believe that there must exist a large amount of sequence and morphological variations of *Myrtama* that cause topological position shift from *Myrtama-Myricaria* clade to *Myrtama-Tamarix* in the two analysis. Based on the variation, *Myrtama elegans* should be put into neither *Myricaria* nor *Tamarix*, but kept in its own monotypic genus, *Myrtama*. In addition, according to ITS sequence characters and the position of *M. elegans* in two generated trees, we consider *Myrtama* an intermediate between *Tamarix* and *Myricaria*, but more closely allied to *Myricaria*. Gaskin *et al.* [5] also noted that *Myrtama* was well supported as a distinct genus from *Myricaria* (100% bootstrap) using 18S, *rbcL*, and tRNA Ser/Gly spacer sequence data, and *Myrtama* was again more closely allied to *Myricaria* than to *Tamarix*.

Morphologically, there are 10 monadelphous stamens in *Myricaria vs*. 4−14 (mainly 4−5) distinct stamens in *Tamarix*, while *Myrtama elegans* has 10 distinct stamens. *Tamarix* tends to have short stylodia, *Myricaria* has sessile stigmas, while *Myrtama elegans* has ten short stigmas. These two characteristics seem to indicate that *M. elegans* more closely resembles *Tamarix* species but bears some variations. Zhang[9] has studied seed morphology of Tamaricace and hypothesized that the tendency of seed evolution is: seed pappus cover half or apex of the seed and all of the coma, nearly a sessile pappus (*Tamarix*), to those cover from bottom of the coma (just with a 0.4mm coma at the apex), nearly almost sessile pappus (*Myrtama*), to those cover only upper part of the coma, nearly stipitate pappus (*Myricaria*). Therefore, *M. elegans* is an intermediate from *Tamarix* to *Myricaria* in this regard.

Additionally, the pollen grains of *Myrtama* under scanning electron microscopy are very different from those of *Myricaria* and *Tamarix* in sculpturing pattern. The *Myrtama* sculpturing is reticulate and the mesh is polygonal and irregular, different from the reticulate ornamentation with circular and regular mesh in *Tamarix*, or non-perforated areolate structure in *Myricaria*[22]. Along with morphological characters, *Myrtama* is also quite unique in chemical constituents. The presence of unidentified amino acid 'C' makes it very distinct from the other genera in Tamaricaceae. However, ellagic acid found in *Tamarix*, and anthocyanadin in *Myricaria*, are both absent in *Myrtama*[8].

In conclusion, the combined morphological, phytochemical and ITS molecular data support the retention of *Myrtama*. Furthermore, *Myrtama* seems to be evolutionary intermediate between *Myricaria* and *Tamarix*, but more closely related to the genus *Myricaria*. So as to the opinion that *Myrtama* is the hybrid genus[12], more molecular evidence should be needed to prove it in the future study.

Acknowledgements The work was supported by the Key Knowledge Innovation Project of the Chinese Academy of Sciences (Grant No. KZCX3-SW-343).

References

1 Crins W J. The Tamaricaceae in the southeastern United States. J Arnold Arb, 1989, 70: 403－425

2 Mabberley D J. The Plant-Book. New York: Cambridge University Press, 1993. 123－130

3 Baum B. The genus Tamarix. Jerusalem: Israel Academy of Sciences and Humanities, 1978. 162－163

4 Zhang P Y, Zhang Y J. Taxonomy study on *Myricaria* Royle in China. Bull Bot Res (in Chinese), 1984, 4(2): 67－80

5 Gaskin J F, Ghahremani-Nejad F, Zhang D Y, et al. An overview of Frankeniaceae and Tamaricaceae using nuclear rDNA and plastid sequence data. Ann Missouri Bot Gard, 2004, 91(3): 401－409

6 Royle J F. Illustration of the botany and other branches of the natural history of the Himalayan mountains and of the flora of Cashmere, London, 1835, 214

7 Ovchinnikov P N, Kinzikaeva G K. *Myrtama* Ovcz. et Kinz. gen nov: the new genus from the family Tamaricaceae Link. Dokl Akad Nauk Tadzh SSR, 1977, 20(7): 54－57

8 Qaiser M, Ali S Z. *Tamaricaria*——A new genus of Tamaricaceae. Blumea, 1978, 24(1): 150－155

9 Zhang Y M, Yin L K, Pan B R. Pollen morphology of the Tamaricaceae from China and its taxonomic significance. Acta Bot Boreal-Occident Sin (in Chinese), 2001, 21(5): 857－864

10 Tang Y C. Xizang Flora (Vo. III) (in Chinese). Beijing: Science Press, 1986. 283－284

11 Xi Y Z. Study on pollen morphology of the Tamaricaceae from China. Bull Bot Res (in Chinese),1988, 8(3): 23－42

12 Wu Z Y, Lu A M, Tang Y C, et al., eds. The Families and Genera of Angiosperms in China (in Chinese). Beijing: Science Press, 2003. 496－499

13 Rogers S O, Bendich A J. Extraction of DNA from plant tissues. Pl Mol Biol Manual, 1988, 6: 1－10

14 White T J, Bruns T, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Sinisky J, et al., eds. Collection in PCR Protocols: A Guide to Methods and Applications. San Diego: Academic Press, 1990. 315－322

15 Takaiwa F, Oono K, Sugiura M. Nucleotide sequence of the 17S-25S spacer region from rice rDNA. Pl Mol Biol, 1985, 4: 355－364

16 Wang X Q, Li Z Y. The application of sequence analysis of rDNA fragment to the systematic study of the subfamily Cyrtandroideae (Gesneriaceae). Acta Phytotax Sin (in Chinese), 1998, 36(2): 97－105

17 Thompson J D, GibsonT J, Plewniak F, et al. The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acid Res, 1997, 25: 4876－4882

18 Kumar S, Tamura K, Jakobsen I B, et al. MEGA: molecular evolutionary genetics analysis, version 2.0. Pennsylvania State University, University Park, and Arizona State University, Tempe, 2000

19 Swofford D L. PAUP* phylogenetic analysis using parsimony, ver 4.0b8. Massachusetts: Sinauer, 2000

20 Huelsenbeck J P, Ronquist F. MRBAYES* Bayesian inference of phylogenetic trees. Bioinformatics, 2001, 17: 754－755

21 Posada D, Crandall K A. Model test: testing the model of DNA substitution. Bioinformatics, 1998, 14(9): 817－818

22 Zhang Y M, Yin L K, Pan B R. Seed morphology characters and its systematic significance of Tamaricaceae in arid zone of China. J Pl Res Environ (in Chinese), 1998, 7(2): 22－27